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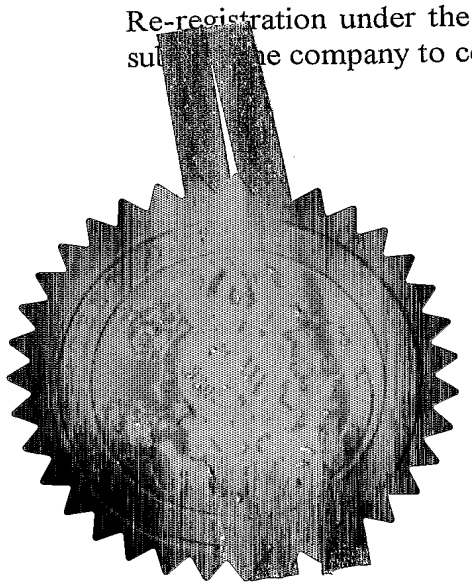

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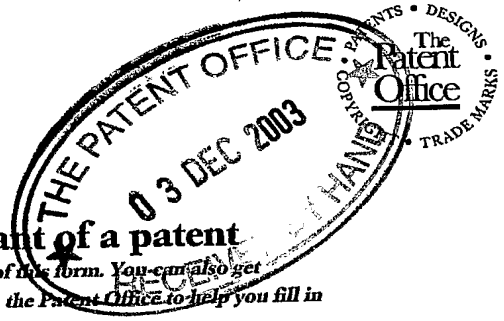

1/77

040EC03 E856918-9 D02890
P01/7700 0.00-0328021.1

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Cardiff Road
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1. Your reference — JWJ01057GB

2. Patent application number *(The Patent Office will fill this part in)* — 0328021.1 — 3 DEC 2003

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Institute of Ophthalmology
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Patents ADP number *(if you know it)* — 5175160003

If the applicant is a corporate body, give the country/state of its incorporation — GB

4. Title of the invention — METHOD

5. Name of your agent *(if you have one)* — Gill Jennings & Every

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Broadgate House
7 Eldon Street
London
EC2M 7LH

Patents ADP number *(if you know it)* — 745002

6. Priority: Complete this section if you are declaring priority from one or more earlier patent applications, filed in the last 12 months.

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. Divisionals, etc: Complete this section only if this application is a divisional application or resulted from an entitlement dispute (see note f)

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8. Is a Patents Form 7/77 (Statement of inventorship and of right to grant of a patent) required in support of this request? — YES

Answer YES if:

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.

Otherwise answer NO (See note d)

Patents Form 1/77

9. Accompanying documents: A patent application must include a description of the invention. Not counting duplicates, please enter the number of pages of each item accompanying this form:

Continuation sheets of this form	
Description	7
Claim(s)	2 CF
Abstract	-
Drawing(s)	6+6

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent (Patents Form 7/77)	
Request for a preliminary examination and search (Patents Form 9/77)	
Request for a substantive examination (Patents Form 10/77)	NO
Any other documents (please specify)	

11. I/We request the grant of a patent on the basis of this application.

For the applicant
Gill Jennings & Every

Signature John Jappy Date 03/12/03

12. Name, daytime telephone number and e-mail address, if any, of person to contact in the United Kingdom

JAPPY, John William Graham
020 7377 1377

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d) If you have answered YES in part 8, a Patents Form 7/77 will need to be filed.

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METHOD

Field of the Invention

The present invention relates to the restoration of visual function by cell transplantation. In particular, the present invention relates to the use of adult retinal stem cells in the treatment of visual disorders.

Background to the Invention

The restoration of visual function is one of the ultimate aims in vision research. Present treatments for severe diseases leading to blindness, such as age related macular degeneration (AMD), glaucoma, diabetic retinopathy and complications of retinal detachment, are only supportive or slow down disease progression, but do not restore visual function. Recent research involving stem cell transplantation in a wide disease spectrum has triggered enthusiasm across the medical and scientific community, and stem cell research to restore neural circuits in diseased retinas are encouraged by results obtained with progenitor cell transplantation to treat other human diseases, including leukemia, severe skin burns and myocardial disfunction.

To date, transplantation studies aimed at restoration of human retinal function have yielded little success, and have been limited to transplantation of retinal pigment epithelial (RPE) and Iris pigment epithelial (IPE) cells. Experimental transplantation of RPE, Schwann and brain derived pre-cursor cells in animal models of retinal degeneration has proved some success in the preservation of retinal function. Retinal transplantation of brain derived precursor cells to RCS rats (a model of retinal degeneration) promotes photoreceptor cell survival. However, although the transplanted cells migrate to the photoreceptor cell layer, they fail to express retinal neural markers, suggesting that a specific neuronal precursor is needed for functional and morphological regeneration of the retina.

During early studies, it was thought that stem cells could only be isolated from embryos, for which neural progenitors were first identified in the embryonic mammalian central system and peripheral nervous system (CNS and PNS). However, more recent investigations have identified adult stem cells in

neurogenic regions of the CNS, and this has prompted further investigations in the search for adult stem cells.

Limb *et al.*, IOVS, 2002; 43(3); 864-869 discloses the identification of a spontaneously immortalised Müller cells.

Müller cells are radial glial cells that extend vertically through the whole width of the retina. They stabilise the complex retinal architecture, give structural and metabolic support to neurons and blood vessels, prevent aberrant photoreceptor migration into the sub-retinal space and regulate fluid transport between the vitreous cavity and the sub-retinal space. Nearly all retinal pathological conditions that constitute major causes of blindness, including age related macular degeneration, proliferative diabetic retinopathy, proliferative vitreoretinopathy (PVR) and retinitis pigmentosa (RP), are associated with changes in Müller cell distribution, proliferation or function.

Fischer *et al.*, Nature Neuroscience, 2001; 4(3): 247-252 describes the identification of Müller glial cells obtained from the retina of post-natal chicken. The Müller glial cells are shown to be non-differentiated, proliferate and express transcription factors normally expressed by embryonic retinal progenitors. The Müller glial cells proliferate in response to retinal damage.

In the human eye, it has been shown that Müller stem cells given origin to various retinal cells are found during foetal development, but no evidence has yet been shown that these cells may be present in the adult neural retina. There is therefore a need to develop cells suitable for use in retinal cell transplantation therapies, for the treatment of human retinal damage.

Summary of the Invention

The present invention is based on the realisation that Müller cells from the adult human neural retina can be obtained and made to behave like stem cells under appropriate conditions.

According to a first aspect of the invention, a method for the production on retinal cells, useful in transplantation therapy, comprises steps of:

(i) obtaining one or more mammalian adult Müller cells; and

(ii) culturing the cells in the presence of an extra-cellular matrix protein and a growth factor, to thereby induce the de-differentiation of the Müller cells into a progenitor phenotype.

The ability to take adult mammalian Müller cells and treat them to induce de-differentiation, allows large quantities of the cells to be obtained and used in transplantation therapy. The cells used according to the invention have been shown to preserve retinal integrity and attenuate loss of visual function when injected into the sub-retinal space of RCS rats.

According to a second aspect of the invention, retinal cells obtainable according to the method outlined above, are used in the manufacture of a medicament for the treatment of a condition associated with cell loss or cell damage in a mammalian eye.

Description of the Invention

The present invention allows for the identification, expansion and maintenance of adult human Müller progenitor cells, permitting the use of the cells in transplantation therapy, to treat various retinal disorders. The adult Müller cells may be isolated from a mammalian donor retina and express markers of mature cells, but on treatment in specific conditions *in vitro*, the cells re-enter the cell cycle and de-differentiate into cells expressing progenitor cell phenotypes such as nestin, βIII tubulin and and binding of peanut agglutinin.

Adult mammalian Müller cells may be obtained from the retina of an adult mammalian eye using techniques disclosed herein. It is preferable to isolate human Müller cells. Under normal culture conditions, the adult Müller cells will express markers of mature Müller cells, including cellular retinaldehyde binding protein (CRALBP), glutamine synthetase, vimentin and epidermal growth factor receptor (EGF-R). The Müller cells are identified by their characteristic morphology under phase-contrast microscopy and by their expression of the markers indicated above. When subconfluent, Müller cells in culture exhibit morphological characteristics typically observed in the retina *in vivo*: they are non-pigmented cells with elongated shape, characteristic end foot processes and villus surfaces; they respond to glutamate as judged by their electrophysiological responses, and do not express GFAP under non-stressed conditions.

In order to produce the de-differentiated Müller cells, it is necessary to culture the isolated adult Müller cells in the presence of an extracellular matrix protein and a growth factor. Extracellular matrix proteins are complex proteins that surround and support cells with an organ. Suitable extracellular matrix proteins will be known to the skilled person, and include matrigel, fibronectin, collagen, vitronectin and laminin. The preferred extracellular matrix proteins are fibronectin and matrigel. The culture conditions also require a growth factor to aid de-differentiation. Suitable growth factors include epidermal growth factor (EGF), fibroblast growth factor-2 (EGF-2), insulin-like growth factor-1 (IGF-1). The preferred growth factor is epidermal growth factor (EGF).

Culturing the adult Müller cells in these conditions allows de-differentiation to occur, leading to Müller cells that express nestin, βIII tubulin and bind peanut agglutinin, which are known markers of embryonic neural cell progenitors.

The de-differentiated cells may be used in this form or may be differentiated into a particular cell phenotype, using specific differentiation agents. For example, in the presence of retinoic acid, a well known differentiating agent of stem cells, the Müller cells form a mosaic of cells resembling ganglion cells and express a low molecular weight neuro filament protein and neuron-specific enolase, characteristic markers of neural cells. Other suitable differentiation agents include 3,3',5-Triiodo-L-thyronine, insulin, and TGFβ. Combinations of differentiating agents may be used to form particular phenotypes. For example, the combination of FGF2 and IGF-1 induce human Müller progenitor cells to express Thy-1, Nueron-specific enolase (NSE) (markers of major retinal neurons such as ganglion cells) and Peripherin (expressed by cones and rod photoreceptor cells).

The differentiation of the Müller cells may also be influenced by the local environment on administration to a patient. In damaged areas, growth factors are produced which may determine the ultimate phenotype of the transplanted Müller cells. The Müller cells may therefore adopt the phenotype of the damaged cells, replacing those lost or damaged cells.

The formation of the de-differentiated Müller cells in the defined culture conditions may be time dependent and it is preferable to culture the isolated Müller cells for at least 2-3 weeks following the addition of the growth factor. Colonies of cells are formed in the culture media and these may be isolated and placed in new culture media to form new cell lines.

The cultured and de-differentiated Müller cells are said to be immortal in that they may be maintained and expanded in culture for multiple passages and retain the ability to differentiate into different phenotypes in response to differentiating agents.

The cells may be used in the treatment of a condition associated with cell loss or cell damage in a mammalian eye. Conditions that may be treated by transplanting the cells of the invention include age-related macular degeneration, proliferative diabetic retinopathy, proliferative vitreoretinopathy, retinal detachment, retinitis pigmentosa, glaucoma and optic nerve injury. Other types of inherited and non-inherited retinal degeneration may also be treated.

The cells used in the treatment of humans should preferably be derived from human cells to reduce problems with immune rejection. The cells may be autologous cells, derived from the mammalian eye to be treated, heterologous cells stored in a cell bank, or genetically modified cell lines derived from these cells.

To treat a patient it is generally of assistance to know where damage has occurred in the eye. Once the existence of damage has been established, whether it be in one isolated area or in several areas, treatment by implantation of cells into the damaged area may be carried out. The cells may be transplanted at a single site, or preferably at multiple sites.

After treatment the progress of the patient may be monitored using tests known to examine cortical visual function. Suitable monitoring techniques include: i) psychophysical tests such as visual field and contrast sensitivity tests, ii) electro-physiological tests such as electro-retinogram (ERG), and iii) high resolution imaging techniques such as ocular coherence tomography (OCT).

Preferably, treatment will substantially correct a visual impairment. However, treatment according to the present invention and with the cells,

medicaments and pharmaceutical preparations of the invention, may lead to improvement in function without complete correction. Such improvement will be worthwhile and of value.

The number of cells to be used will vary depending on the nature and extent of damage. Typically, the number of cells used in transplantation will be in the range of about 100,000 to several million. Treatment need not be restricted to a single transplant. Additional transplants may be carried out to further improve function. The cells prepared according to the invention may be formulated with any pharmaceutically acceptable diluent or excipient and may include additional pharmaceutical agents, including immunosuppressive agents or growth factors. The cells may also be genetically modified to express other pharmaceutical agents which may be required at the site of damage.

The cells may also be combined with agents known to plasticize the nervous system, which may enhance the ability of the cells to connect to the nervous system and to grow into the eye.

The following example illustrates the invention.

Example

1. De-differentiation of adult Müller progenitor cells into cells expressing markers of retinal neurons:

Müller cells in suspension were cultured on matrigel coated culture plates at a density of 1000 cells/ml in DMEM medium containing 10% foetal bovine serum (FCS) and either FGF-2 (40 µg/ml) alone or in combination with insulin (100 µg/ml), ii) retinoic acid (500 nM), or iii) triiodothyronine (T3, 40 µg/ml). Following 3-10 days in culture with medium freshly replaced every 48 hours, cells exhibited various changes in morphology and expression of neural retinal markers. For example, following 5 days in culture on Matrigel and in the presence of FGF2, cells formed neurospheres and cells contained in the neurospheres bound PNA and expressed nestin, calretinin and βIII tubulin (markers of neural progenitors). When cultured on fibronectin in the presence of retinoic acid, Müller cells formed neurospheres and also displayed neural morphology. They also expressed nestin and markers of retinal neurons including 68kD neurofilament protein, thy-1 and calretinin, as well as rhodopsin,

a marker of rod photoreceptor cells. Following 10 days in culture on matrigel in the presence of FGF2 and IGF-1, they displayed neural morphology and expressed the retinal neural markers thy-1, neuron-specific enolase and calbinding. In addition, they expressed rhodopsin and peripherin, both markers of photoreceptor cells.

2. Restoration of visual function in an experimental models of retinal degeneration

Three week-old dystrophic Royal College of Surgeons (RCS) rats, which exhibit progressive photoreceptor degeneration accompanied by ganglion cell loss, were immunop-suppressed with cyclosporine A and injected via a trans-scleral route into the dorso-temporal subretinal space with 2 µl of DMEM medium containing 10^5 Müller cells. Retinal architecture and localization of the transplanted cells were examined at 8, 12 and 15 weeks after transplantation using histo-pathological and immuno-histochemical techniques. Visual function was measured by timing the animals' head movement in a set interval, when placed on a stationary platform in the centre of a rotating drum lined with black and white stripes. Using this experimental protocol, if the rat can see, the moving lines evoke an involuntary optokinetic response such as the rat track the movement. The results showed that human Müller stem cells transplanted into the subretinal space of RCS rats migrated across the retina and localized into the photoreceptor and ganglion cell layers.

Examination of retinal morphology in the transplanted rats showed that the photoreceptor cell layer and general retinal appearance were well preserved. They also showed that transplanted Müller stem cells preserved visual function in the RCS rat, as judged by head tracking responses.

CLAIMS

1. A method for the production of retinal cells, useful in transplantation therapy, comprising the steps of:

(i) obtaining one or more mammalian adult Müller cells; and

(ii) culturing the cells in the presence of an extracellular matrix protein and a growth factor to thereby induce dedifferentiation of the Müller cells into a progenitor phenotype.

2. A method according to claim 1, wherein the extracellular matrix protein is fibronectin and the growth factor is EGF.

3. A method according to claim 1 or claim 2, wherein the cells are human Müller cells.

4. A method according to any preceding claim, wherein the dedifferentiated cells are further cultured in the presence of an extracellular matrix protein and differentiation agents, to thereby induce the dedifferentiated cells to adopt a specific differentiated cell phenotype.

5. A method according to claim 4, wherein the extracellular matrix is matrigel, fibronectin, collagen or laminin, and the differentiation agents are FGF-2, retinoic acid, 3,3',5-Triiodo-L-Thyronine, insulin, insulin-like growth factor or TGFβ.

6. A composition comprising cells obtainable by a method as defined in any preceding claim.

7. Use of a retinal cell obtainable by a method as defined in any of claims 1 to 5, in the manufacture of a medicament for the treatment of a condition associated with cell loss or cell damage in a mammalian eye.

8. Use according to claim 7, wherein the cell is a human cell.

9. Use according to claim 7 or claim 8, wherein the retinal cell is a pluripotent adult Müller cell.

10. Use according to any of claims 7 to 9, wherein the condition to be treated is selected from the group consisting of: age-related macular degeneration, proliferative diabetic retinopathy, proliferative vitreoretinopathy, retinal detachment, retinitis pigmentosa, glaucoma and optic nerve injury and degeneration.

11. Use according to any of claims 7 to 10, wherein the cells are autologous cells, derived from the Mammalian eye to be treated, heterologous cells stored in a cell bank, or genetically modified cells derived from the mammalian eye or cell bank.

Characteristic Müller cell morphology

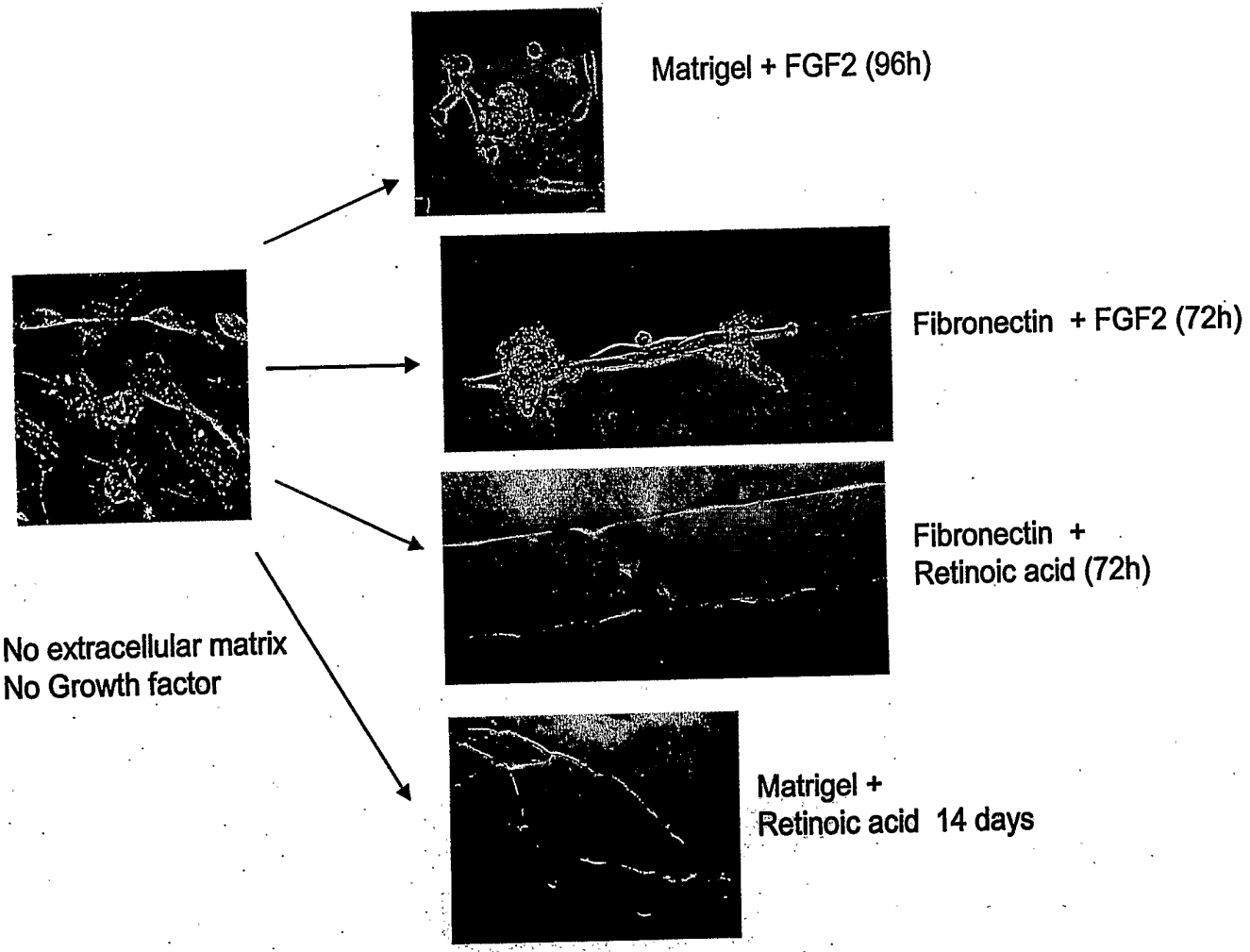
Appearance of Müller cells in
plastic tissue culture dishes

A

B

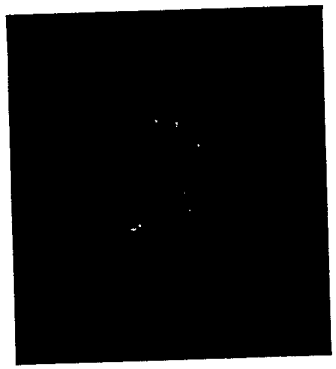
Scanning microscopy images of Müller cells
cultured on plastic tissue culture plates showing
A. Characteristic microvilli and **B.** end foot processes

Morphological changes of human Müller cells under different culture conditions

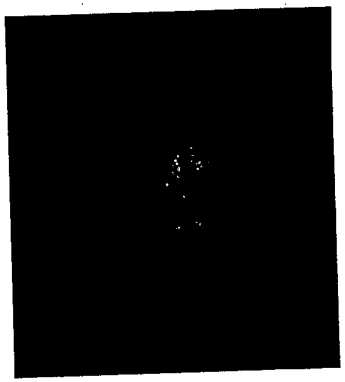


Formation of embryoid bodies by Müller cells cultured under various conditions

A. Expression of Cyclin D

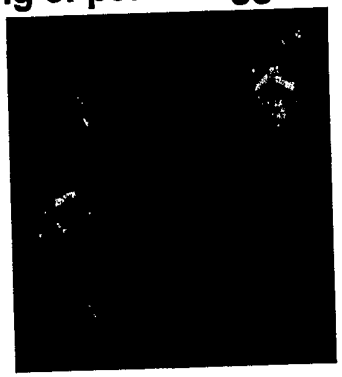


Fibronectin +
FGF2 + Insulin (5 days)

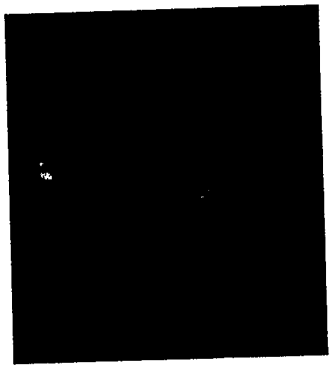


Matrigel + FGF2
(5 days)

B. Binding of peanut agglutinin

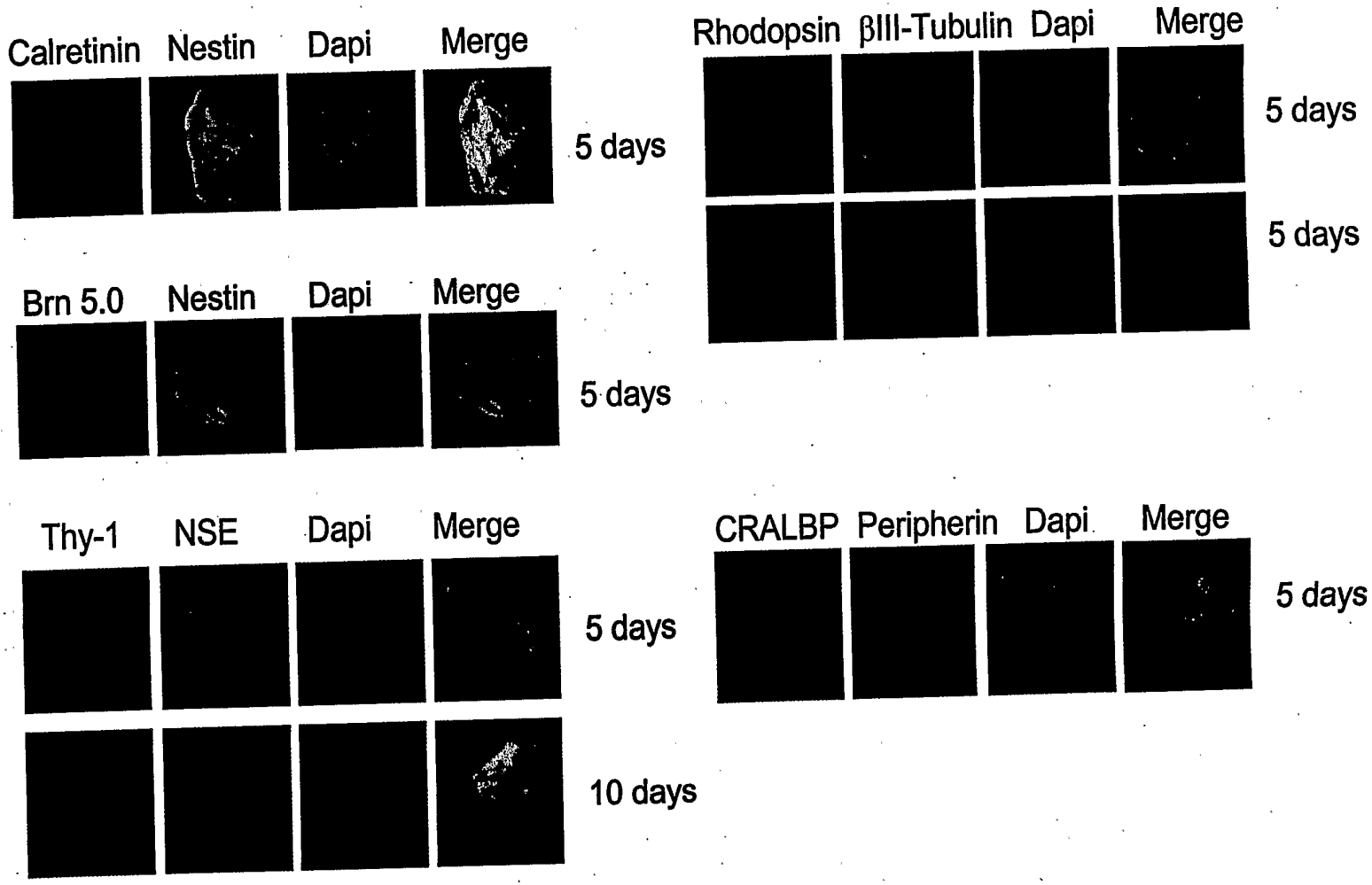


Fibronectin + FGF2 (5 days)

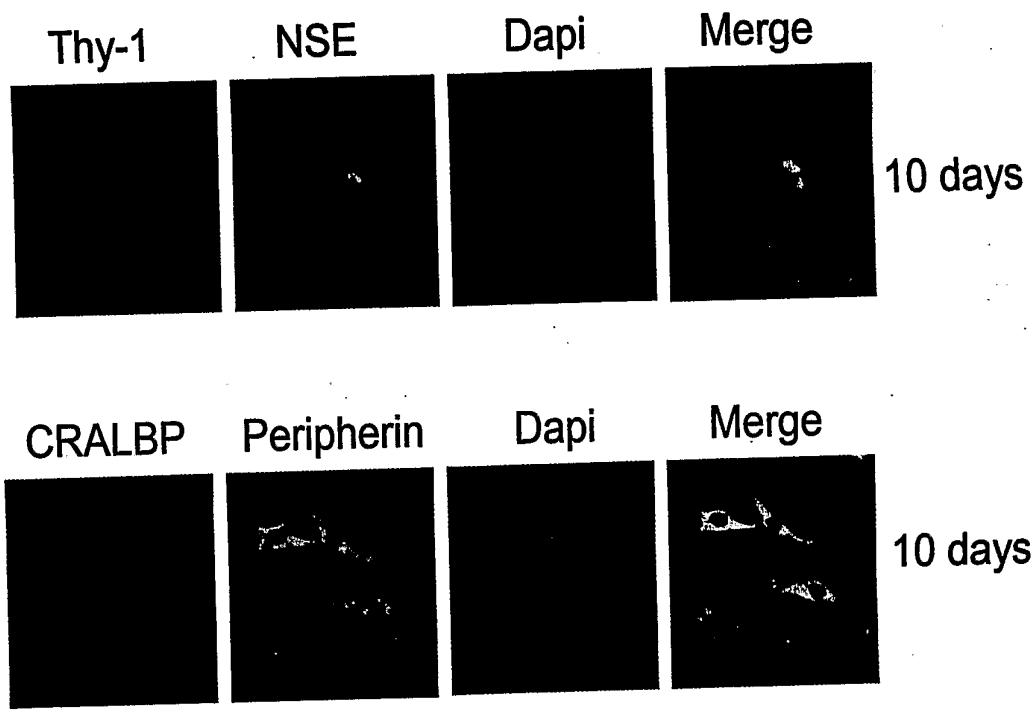


Matrigel + Retinoic
acid (5 days

Expression of retinal neural markers by Müller cells cultured on matrigel in the presence of retinoic acid

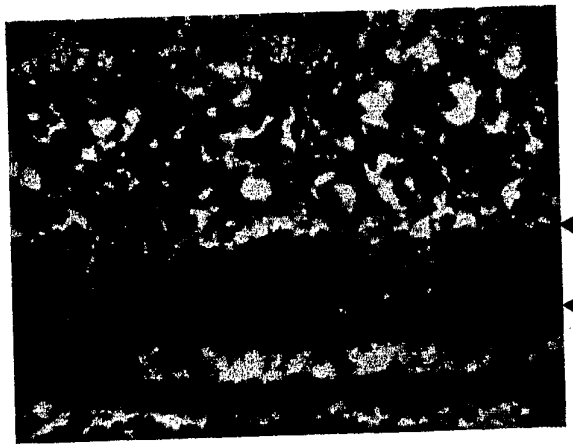


Expression of neural retinal markers by Müller cells cultured on matrigel in the presence of FGF2 + IGF-I



Müller cell transplant in RCS rats: retinal appearance at 4 months

Untreated eye



Loss of photoreceptor cells

Cell debris

Eye transplanted With Müller cells

Preservation of photoreceptor cells

Minimal cell debris